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Cloning, Characterization, and Functional Expression of *acs*, the Gene Which Encodes Acetyl Coenzyme A Synthetase in *Escherichia coli*

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Acetyl coenzyme A synthetase (Acs) activates acetate to acetyl coenzyme A through an acetyladenylate intermediate; two other enzymes, acetate kinase (Ack) and phosphotransacetylase (Pta), activate acetate through an acetyl phosphate intermediate. We subcloned *acs*, the *Escherichia coli* open reading frame purported to encode Acs (F. R. Blattner, V. Burland, G. Plunkett III, H. J. Sofia, and D. L. Daniels, Nucleic Acids Res. 21:5408–5417, 1993). We constructed a mutant allele, Δ*acs*::Km, with the central 0.72-kb *Bcl*I-*Bcl*I portion of *acs* deleted, and recombined it into the chromosome. Whereas wild-type cells grew well on acetate across a wide range of concentrations (2.5 to 50 mM), those deleted for *acs* grew poorly on low concentrations (≤10 mM), those deleted for *ackA* and *pta* (which encode Ack and Pta, respectively) grew poorly on high concentrations (≥25 mM), and those deleted for *acs*, *ackA*, and *pta* did not grow on acetate at any concentration tested. Expression of *acs* from a multicopy plasmid restored growth to cells deleted for all three genes. Relative to wild-type cells, those deleted for *acs* did not activate acetate as well, those deleted for *ackA* and *pta* displayed even less activity, and those deleted for all three genes did not activate acetate at any concentration tested. Induction of *acs* resulted in expression of a 72-kDa protein, as predicted by the reported sequence. This protein immunoreacted with antiserum raised against purified Acs isolated from an unrelated species, *Methanothrix soehngenii*. The purified *E. coli* Acs then was used to raise anti-*E. coli* Acs antiserum, which immunoreacted with a 72-kDa protein expressed by wild-type cells but not by those deleted for *acs*. When purified in the presence, but not in the absence, of coenzyme A, the *E. coli* enzyme activated acetate across a wide range of concentrations in a coenzyme A-dependent manner. On the basis of these and other observations, we conclude that this open reading frame encodes the acetate-activating enzyme, Acs.

Escherichia coli cells activate acetate to acetyl coenzyme A (acetyl-CoA) by two distinct pathways (Fig. 1). One pathway, catalyzed by the enzyme acetyl-CoA synthetase (Acs) (acetate: CoA ligase [AMP forming]; EC 6.2.1.1), proceeds through an acetyladenylate (AcAMP) intermediate (3, 8). The second pathway, catalyzed by the enzymes acetate kinase (Ack) [acetyl-CoA(CoA):P_i acetyltransferase; EC 2.7.2.1] and phosphotransacetylase (Pta) (ATP:acetate phosphotransferase; EC 2.3.1.8), proceeds through an acetyl phosphate intermediate (41). Acs first converts acetate and ATP to AcAMP, producing PP_i; it then reacts AcAMP with CoA to form acetyl-CoA, releasing AMP. Similarly, Ack first converts acetate and ATP to acetyl phosphate, producing ADP; Pta then reacts acetyl phosphate with CoA to form acetyl-CoA, releasing P_i.

Brown et al. (6) hypothesized that the Acs pathway functions as a catabolite-repressible, acetate-inducible, high-affinity acetate uptake system that scavenges acetate present extracellularly at relatively low concentrations. They also proposed that the Ack-Pta pathway functions primarily in a catabolic role, excreting acetate and generating ATP during mixed-acid fermentation and aerobic growth on excess glucose or other glycolytic intermediates. Finally, they argued that the low-affinity Ack-Pta pathway activates acetate only when that molecule is present extracellularly in large quantity.

In addition to their role in acetate metabolism, the acetate activation pathways have been implicated in the regulation of signal transduction by two-component regulatory systems in several bacterial species (reviewed in references 32 and 54; see also references 2, 9, 39, and 55), the regulation of the glucose starvation stimulon of *E. coli* (35), and the synthesis of adenosine 5′-tetraphosphate and adenosine 5′-pentaphosphate in *Saccharomyces cerevisiae* (17).

Although *E. coli* mutants deficient for Ack and Pta activities have existed for some time, those deficient for Acs activity have not. Recently, Blattner et al. (5) reported an open reading frame, located within min 92 of the *E. coli* chromosome, that bears significant homology to those which encode Acs in other organisms. We now report the subcloning of the putative *E. coli acs* gene, the construction and characterization of mutant cells deleted for a portion of that gene, and the purification of its protein product. On the basis of a variety of biological, immunological, and biochemical assays, we conclude that this open reading frame does indeed encode Acs and that *E. coli* cells require both acetate activation pathways for optimal growth across a wide range of acetate concentrations.

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MATERIALS AND METHODS

Chemicals. The enzymes and substrates used in the enzyme assays were obtained from Boehringer Mannheim (Indianapolis, Ind.) or Sigma Chemical Company (St. Louis, Mo.). Radiolabelled materials were purchased from Amersham

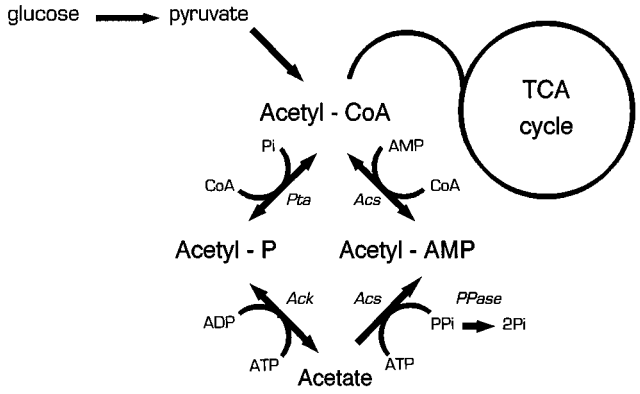

FIG. 1. Pathways of acetate activation in *E. coli*. Acetyl-P, acetyl phosphate; acetyl-AMP, AcAMP; PPase, pyrophosphatase.

(Arlington Heights, Ill.), and the bicinchoninic acid protein assay reagent was obtained from Pierce Biochemicals (Rockford, Ill.).

Plasmids. All plasmids used in this study are represented in Fig. 2. pSR6 and pSR9 were constructed by subcloning the 2.2-kb *Mun*I-*Mun*I restriction fragment of λ638 (24) in both orientations into the unique *Eco*RI site of pUC19 (53). pSR8 was constructed by deleting the 0.72-kb *Bcl*I-*Bcl*I restriction fragment of pSR6 and replacing it with the 1.3-kb *Bam*HI-*Bam*HI fragment of pUC4K (Pharmacia Biotech, Piscataway, N.J.) which confers kanamycin resistance. This deletion is referred to as Δ*acs*::Km. pSR10 was constructed by subcloning the same 2.2-kb *Mun*I-*Mun*I restriction fragment of λ638 into the unique *Eco*RI site of pRS415 (45). This resulted in an operon fusion such that the putative *acs* promoter, P_{acs}, should control the expression of Acs, β-galactosidase, and lactose permease.

pSR30 and pSR31 were constructed by subcloning the 2.14-kb *Sph*I-*Sph*I restriction fragment of pSR9 into the unique *Sph*I site of pALTER-1 (Promega, Madison, Wis.). The translational start site was converted by site-directed mutagenesis to an *Nde*I restriction site which, in conjunction with a *Bam*HI site located downstream of the translational termination site, then was used to subclone the open reading frame into pET-14b (Novagen Inc., Madison, Wis.), yielding pSR30. This plasmid permitted expression, by means of the bacteriophage T7 φ10 promoter, of a His-Tag sequence fused to the N terminus of Acs. The *Nde*I-*Bam*HI restriction fragment also was subcloned into pUC19, yielding pSR31, a plasmid that expressed *acs* under the control of the lactose promoter, P_{lac}.

Bacterial strains. All strains were derivatives of *E. coli* K-12 and are listed in Table 1. The *acs* deletion, Δ*acs*::Km, was introduced into the chromosome by means of homologous recombination (36) in the PolA(Ts) host strain AJW763 following transformation (44) with pSR8. Generalized transduction, involving the use of the phage P1*kc* (44), was used to transfer the mutant allele, Δ*acs*::Km, from its PolA(Ts) host into the $PolA^+$ Rec^+ Acs^+ recipient strains CP875 and CP911, to yield strains AJW803 and AJW804, respectively. P1 transduction also was used to make the recombination-deficient, acetate activation-deficient strain AJW807. Strains BMH71-18*mutS*, DH5α, and JM109 were used for standard molecular biological techniques.

Media and growth conditions. Cells were grown with aeration in tryptone broth (TB), composed of 1% (wt/vol) tryptone and 0.5% (wt/vol) sodium chloride; in Luria broth (LB), composed of TB and 0.5% (wt/vol) yeast extract; or in M63 minimal salts medium (34), containing glycerol and/or acetate at the concentrations indicated below. The optical density at 610 nm (OD_{610}) was monitored. When crude lysates were required, cells were harvested by centrifugation at 3,000 × *g* for 20 min at 4°C, washed with phosphate-buffered saline (PBS), and lysed by sonication.

Determination of extracellular acetate concentration. Cells were grown in TB, aliquots were removed at appropriate intervals, the cells were removed by centrifugation, and the supernatant liquid (1 ml) was assayed for acetate in a coupled reaction as described elsewhere (4).

Determination of acetate-activating activity. Crude lysates prepared by sonication were centrifuged at 14,000 × *g* for 1 h at 4°C to remove cellular debris and then dialyzed against 20 mM Tris · Cl (pH 8.0) for 3 h at 4°C to remove small molecules. The acetate-activating activity of crude extracts or purified protein was measured by using the hydroxylamine assay of Jones and Lipmann (22) as described by Brown et al. (6). This assay makes use of the fact that in the presence of hydroxylamine, activated derivatives of acetate form acetohydroxamate, which then can be measured photometrically with $FeCl_3$ against a blank consisting of all reaction components with the exception of acetate. Specific activities were expressed in terms of milliunits per milligram, where 1 mU of activity catalyzes the formation of 1 nmol of acetohydroxamate per min. Protein concentrations were determined by the bicinchoninic acid reagent method (46).

Protein purification. Acs was purified by transforming pSR30, the plasmid which expresses the His-Tag–Acs fusion protein under the control of the T7 promoter, into BL21(λDE3), a strain which expresses T7 RNA polymerase under the control of P_{lac} (50). Cells of the resultant transformant were grown to an OD_{610} of ~0.4 at 37°C in LB containing carbenicillin (100 μg/ml) before expression of the fusion protein was induced by the addition of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Following a 3-h incubation under induc-

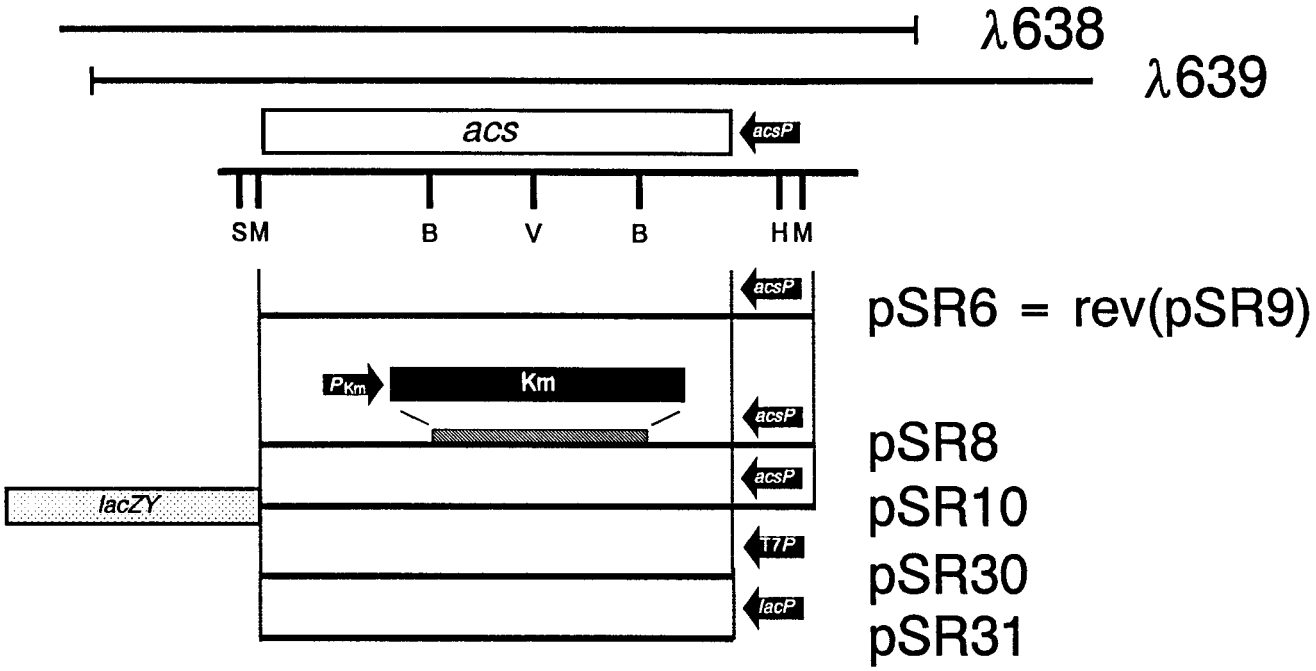

FIG. 2. Bacteriophages and plasmids used in this study that encode Acs. Endpoints are indicated by the restriction sites, which were either present in the native sequence or prepared by site-directed mutagenesis. B, *Bcl*I; H, *Sph*I; M, *Mun*I; S, *Stu*I; and V, *Eco*RV. The cassette that confers kanamycin resistance (Km), the deleted region (hatched bar), and relevant promoters and their orientations (arrows) are indicated.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype[a]	Source or reference
AJW377	*cheA503I*(Am) *recA*::Cm *thi-1 thr-1*(Am) *leuB6 metF159*(Am) *his-4 rpsL136 lacY1 xyl-5 ara-14 tonA31 tsx-78*	56
AJW763	*polA*(Ts) *rha zig*::Tn*10 thi-1 thr-1*(Am) *leuB6 metF159*(Am) *his-4 rpsL136 lacY1 xyl-5 ara-14 tonA31 tsx-78*	This study
AJW803	CP875 Φ(Δ*acs*::Km-1)	This study
AJW804	CP875 Φ(Δ*acs*::Km-1) Δ(*ackA pta hisJ hisP dhu*)	This study
AJW807	AJW804 *recA*::*cml*	This study
AJW810	AJW807 pSR31	This study
AJW811	AJW807 pSR10	This study
BL21(λDE3)	*hsdS gal ompT* r_B^- m_B^- [λ*c*I857(Ts) *ind1 S*am*7 nin5 lacUV5*-T7 gene *1*]	50
BMH71-18*mutS*	*thi supE* Δ(*lac-proAB*) *mutS*::Tn*10*/F′ *proA*$^+$*B*$^+$ *lacI*^{q}*Z*ΔM15	26
CP875	Δ*lacX74 thi-1 thr-1*(Am) *leuB6 metF159*(Am) *rpsL136* λ*lacY*	39
CP911	CP875 Δ(*ackA pta hisJ hisP dhu*)	39
DH5α	*supE44* Δ(*argF-lac*)*U169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	18
JM109	*supE44* Δ(*lac-proAB*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	58
SE3001	Δ(*malK-lamB*)*1 araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flhD5301 deoC1 ptsF25 rbsR*	44

[a] Δ(*ackA pta hisJ hisP dhu*) is linked to *zej*::Tn*10*, which confers Tc^r.

ing conditions, the cells were harvested by centrifugation at 3,000 × *g* for 20 min at 4°C. Purification of the fusion protein and cleavage of His-Tag were performed according to the manufacturer's instructions (Novagen [20]) except that CoA (533 μM) was added to the elution and storage buffers to ensure that the purified protein retained activity.

SDS-polyacrylamide gel electrophoresis and immunoblotting. A 50-μg sample of total protein was loaded onto a 10% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS) and subjected to a current of 30 mA at 4 to 10°C in a Bio-Rad minigel apparatus (27) until the loading dye was about 1 cm from the bottom. The proteins were transferred electrophoretically overnight to a 0.45-μm-pore-size nitrocellulose filter by using a Trans-Blot Cell (Bio-Rad, Hercules, Calif.). The nitrocellulose was blocked with 5% (wt/vol) nonfat dried milk in PBST (0.01 M sodium phosphate [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature. The filter was washed for 5 min in PBST before being subjected to sequential incubation with rabbit anti-*Methanothrix soehngenii* Acs (a gift from R. Eggen, Wageningen Agricultural University, Wageningen, The Netherlands) or rabbit anti-*E. coli* Acs (obtained by standard procedures [19]) for 1 h at room temperature and goat anti-rabbit immunoglobulin G (heavy- and light-chain specific) at appropriate dilutions for 1 h at room temperature. Finally, it was washed three times for 5 min each in PBST between each incubation step. Color development was achieved with 0.5 mg of 4-chloro-1-naphthol ml^{-1} in PBS with 17% (vol/vol) methanol at a final concentration of 0.015% H_2O_2.

Southern blot analysis. DNA-DNA hybridization was performed by the method of Southern (47) as described by Davis et al. (10). Two probes, the 0.72-kb *Bcl*I-*Bcl*I fragment of pSR9 (Fig. 2) and the 1.3-kb *Bam*HI-*Bam*HI kanamycin cassette derived from pUC4K, were labelled with [^{32}P]dCTP by using the Ready-To-Go DNA labelling kit (Pharmacia). The 0.72-kb *Bcl*I-*Bcl*I probe was used to hybridize the *E. coli* Gene Mapping Membrane (TaKaRa Shuzo Co., Ltd.), which represents the entire *E. coli* chromosome. The 0.72-kb *Bcl*I-*Bcl*I and 1.3-kb *Bam*HI-*Bam*HI probes also were used to hybridize chromosomal DNA digested with either *Eco*RV or *Eco*RI. After hybridization for 16 h at 42°C, the blots were exposed to X-ray film at −70°C for 24 h.

Sequence analysis. Double-stranded DNA was purified with the Magic Miniprep purification system (Promega) and denatured as described elsewhere (7). Single-stranded DNA was prepared by the method of Messing (33) with the Magic M13 DNA purification system (Promega). Both single- and double-stranded DNAs were sequenced by the chain termination method (43) using Sequenase, version 2.0 (U.S. Biochemicals, Cleveland, Ohio). Sequence analysis was performed with the software package (version 7.2) of the Genetics Computer Group (13a).

RESULTS

Genetic and molecular characterization. To determine whether the putative *acs* open reading frame actually encodes Acs (5), we subcloned the 2.2-kb *Mun*I-*Mun*I restriction fragment of the recombinant phage λ638 of Kohara et al. (24) into pUC19 (53) to yield the plasmid pSR9. To verify the identity of the 2.2-kb *Mun*I-*Mun*I region of pSR9, we performed restriction enzyme analysis using seven different enzymes and sequenced about 300 bases from each of the 5′ and 3′ ends of the fragment. The results of both analyses corresponded identically to those predicted by the sequence of Blattner et al. (5).

To provide genetic evidence that the putative *acs* open reading frame encodes Acs, we constructed the deletion allele Δ*acs*::Km and recombined it into the chromosome of the PolA(Ts) strain AJW763. To determine whether Δ*acs*::Km had recombined into the 92-min region of the chromosome, we mapped it relative to the neighboring genes, *malK* and *lamB*, which contribute to the ability of cells to utilize maltose as their sole carbon source and which map to the 91.5-min region (42). To do so, we used cells of the putative Acs-deficient strain, AJW803, as the source of donor DNA to P1 transduce cells of the LamB- and MalK-deficient strain, SE3001, selecting for transductants resistant to kanamycin. We then screened kanamycin-resistant transductants for the ability to grow on minimal media supplemented with maltose (0.2%). The phenotypes Km^r and Mal$^+$ cotransduced with a frequency of about 25% (46 of 179 Km^r transductants were also Mal$^+$), indicating that Δ*acs*::Km mapped to the 90- to 93-min region.

Wu et al. (57) reported the sequence of a partial open reading frame called *orfX*, located 3′ to *ubiG*, that shares about 1 kb of 100% identity with that reported by Blattner et al. (5). Whereas *ubiG* maps within the 50-min region of the chromosome (42), Blattner and coworkers located the putative *acs* open reading frame within the 92-min region. To distinguish between these two possible locations, we used the 0.72-kb *Bcl*I-*Bcl*I restriction fragment of pSR9 to probe the library of recombinant bacteriophages of Kohara et al. (24). This 0.72-kb restriction fragment, which was deleted to construct the Δ*acs*::Km allele, hybridized only with phages which carried the 92.3- to 92.4-min region, i.e., λ638 and λ639 (data not shown).

To determine whether the resultant recombinants carried a single putative *acs* allele, we performed a Southern blot analysis. The 0.72-kb *Bcl*I-*Bcl*I probe (Fig. 3A) hybridized to 4.5- and 2.5-kb *Eco*RV (lane 1) and 17.3-kb *Eco*RI (lane 3) chromosomal fragments of the wild-type strain, CP875; it did not hybridize with chromosomal fragments of the Δ*acs*::Km strain, AJW803 (lanes 2 and 4). In contrast, the 1.3-kb *Bam*HI-*Bam*HI probe (Fig. 3B), derived from plasmid pUC4K and used to mark the *acs* deletion, did not hybridize to chromosomal fragments of strain CP875 (lanes 1 and 3). However, it did hybridize to 7.6-kb *Eco*RV (lane 2) and 17.9-kb *Eco*RI (lane 4) chromosomal fragments from strain AJW803.

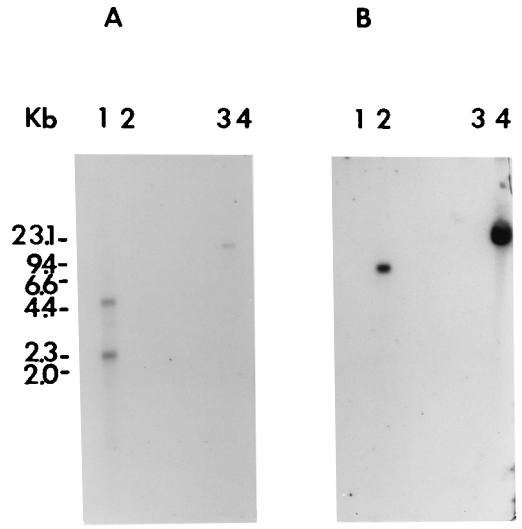

FIG. 3. Southern hybridization analysis demonstrating the location of the kanamycin-resistant deletion allele Δ*acs*::Km. Chromosomal DNA, isolated from cells wild type for acetate activation (strain CP875) or their Δ*acs*::Km derivatives (strain AJW803), was digested with *Eco*RV or *Eco*RI, hybridized with the 0.72-kb *Bcl*I-*Bcl*I fragment of pSR9 (A), stripped, and rehybridized with the 1.1-kb *Bam*HI-*Bam*HI fragment of pUC4K (B). Lanes 1 and 2, *Eco*RV digests of DNA derived from strains CP875 and AJW803, respectively; lanes 3 and 4, *Eco*RI digests of DNA from strains CP875 and AJW803, respectively. Fragment sizes (in kilobases) are indicated on the left.

Phenotypic characterization. To characterize the phenotype of cells deleted for *acs*, we grew wild-type (strain CP875) cells or cells deficient for either Acs (strain AJW803) or Ack and Pta (strain CP911) at 37°C in TB to mid-exponential phase (OD_{610}, ~0.3), washed and diluted them 10^{-5} in PBS (pH 7.4), plated them onto M63 minimal salts medium supplemented with a range of acetate concentrations (0 to 50 mM) as the sole carbon source, incubated them at 37°C for 60 h, and compared the resultant colony sizes (Fig. 4). Wild-type cells grew relatively well at all concentrations of acetate tested. Although cells of the putative Acs-deficient strain grew about as well as wild-type cells at higher concentrations (≥25 mM), they grew poorly on lower concentrations (≤10 mM). In contrast, cells of the Ack- and Pta-deficient strain grew best at lower concentrations (≤10 mM). Finally, cells deficient for Acs and Ack, Acs and Pta, or Acs, Ack, and Pta did not grow on any of the concentrations tested (data not shown).

We inoculated TB with wild-type (strain CP875) cells or cells deficient for Acs (strain AJW803), Ack and Pta (strain CP911), or Acs, Ack, and Pta (strain AJW804), aerated the cultures at 37°C, monitored their growth rates by optical density, and measured their extracellular acetate concentrations (Fig. 5). Wild-type and Acs-deficient cells grew at similar rates (~1.7 generations per h) and excreted similar amounts of acetate (~1.2 mM) prior to mid-exponential phase (OD_{610}, ≤0.4). Although the two cell types continued to grow at similar rates, their behavior with respect to extracellular acetate differed. Whereas the concentration of extracellular acetate of wild-type cells declined to a minimum of <0.1 mM, that of the Acs-deficient cells did not decrease. In contrast, the growth of cells

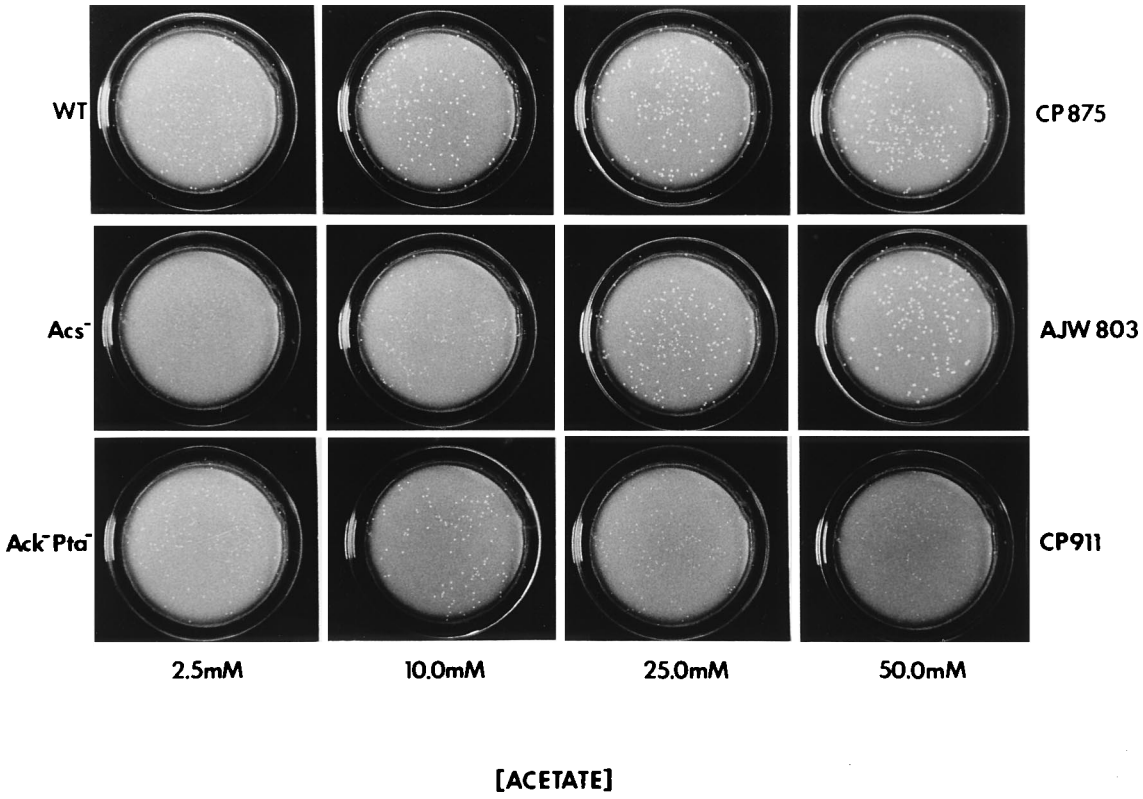

FIG. 4. Growth on acetate as the sole carbon source of cells wild type for acetate activation (strain CP875) or deficient for either Acs (strain AJW803) or Ack and Pta (strain CP911). Cells were grown at 37°C in TB to an OD_{610} of ~0.3, washed and resuspended in PBS, spread onto M63 minimal plates supplemented with the indicated concentration of acetate, incubated at 37°C for 72 h, and photographed.

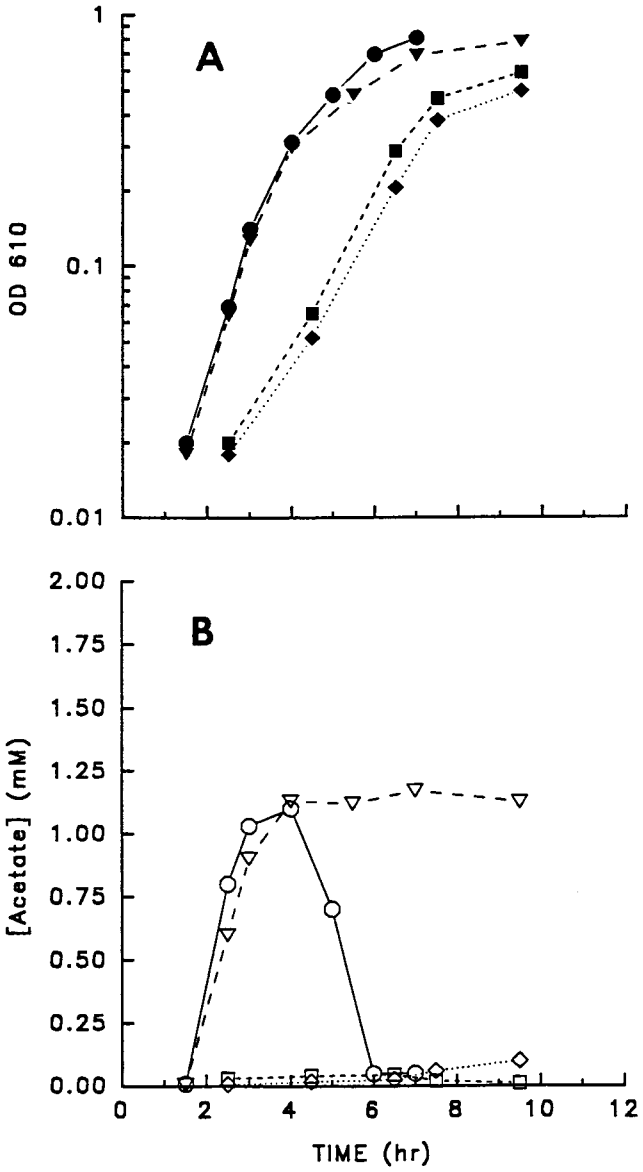

FIG. 5. Optical density (A) and extracellular acetate concentration (B) plotted as a function of time for cells wild type for acetate activation (● and ○; strain CP875) or deficient for Acs (▼ and ▽; strain AJW803), Ack and Pta (■ and □; strain CP911), or Acs, Ack, and Pta (◆ and ◇; strain AJW804) grown in TB at 37°C.

deficient for Ack and Pta or for Acs, Ack, and Pta lagged substantially (between 2 and 3 h) before somewhat lower rates of growth (~1.4 generations per h) were attained. Neither cell type excreted appreciable amounts of acetate.

Next, we attempted to rescue growth on acetate of cells deficient for Acs, Ack, and Pta. We transformed cells deficient for Acs, Ack, Pta, and RecA (strain AJW807) with plasmids which expressed *acs* under the control of P_{acs} (pSR10) or P_{lac} (pSR31) to yield strains AJW811 and AJW810, respectively. We grew cells of the parental and transformant strains at 37°C in TB to mid-exponential phase (OD_{610}, ~0.3), washed and diluted them with PBS, and plated them onto M63 minimal medium supplemented with acetate (10 mM) and a range of IPTG concentrations (0 to 200 μM). After 60 h of incubation at 37°C, the plates were removed and the resultant colony sizes were compared (Fig. 6). In contrast to cells which expressed Acs from its native promoter, P_{acs} (strain AJW811), those of the parental strain (AJW807) did not grow. Cells which expressed Acs from the IPTG-inducible promoter, P_{lac} (strain AJW810), formed colonies only when grown in the presence of 25 to 100 μM IPTG, forming the largest colonies in the presence of 50 μM IPTG. They did not form colonies in the absence of IPTG or in the presence of 200 μM IPTG (data not shown).

Enzymatic and immunological characterization. To test for Acs activity, we grew wild-type (strain CP875) cells or cells deficient for Acs (strain AJW803), Ack and Pta (strain CP911), or Acs, Ack, and Pta (strain AJW804) at 37°C in either TB or M63 minimal medium supplemented with acetate and glycerol. At various times, we harvested and washed cells and prepared crude lysates which were centrifuged and dialyzed to remove cellular debris and small molecules and assayed for the ability to convert acetate to one of its activated forms.

When grown in TB, cells which were wild type (strain CP875) or deficient for Acs (strain AJW803) activated acetate (present in the reaction mixture at 2.5, 5.0, or 10.0 mM) across the range of optical densities tested, i.e., OD_{610}s of 0.2, 0.4, and 0.6 (Table 2). Cells deficient for Ack and Pta (strain CP911), however, activated acetate only upon passing through mid-exponential phase (OD_{610} = 0.4). This activity was significantly reduced relative to those exhibited by wild-type cells and cells deficient for Acs. In contrast, cells deficient for all three enzymes (strain AJW804) exhibited no detectable acetate-activating activity.

Under all conditions tested, the activity of Ack and Pta seemed to be partially dependent on the presence of CoA. For example, when CoA was excluded from the reaction mixture, the activity of cells which expressed either Acs, Ack, and Pta (strain AJW875) or Ack and Pta (strain AJW803) decreased, on average, by about 70% relative to that observed when CoA was included. In contrast, the activity of Acs was completely dependent on the presence of CoA. Cells that expressed only Acs exhibited no detectable activity in the absence of CoA (data not shown).

When grown overnight in M63 minimal medium supplemented with acetate (50 mM) and glycerol (2.5 mM), cells which were wild type (strain CP875) or deficient for either Acs (strain AJW803) or Ack and Pta (strain CP911) activated acetate (present in the reaction mixture at 2.5, 5.0, or 10.0 mM; Table 3). Wild-type cells exhibited the highest activity, while cells deficient for Ack and Pta displayed the lowest. Cells deficient for Acs, i.e., those that expressed Ack and Pta, increased their activity relative to acetate concentration. In contrast, cells deficient for Ack and Pta, i.e., those that expressed Acs, did not change their activity relative to acetate concentration. Under all conditions tested, the activity exhibited by wild-type cells was approximately equal to the sum of the Ack-Pta and Acs activities exhibited by cells deficient for Acs or for Ack and Pta, respectively. In contrast, cells deficient for all three enzymes (strain AJW804) exhibited no detectable acetate-activating activity.

To verify the presence or absence of Acs expression in wild-type strains or strains deficient for Acs, we required a polyclonal antibody specific for *E. coli* Acs. To prepare such an antibody, we purified, to >95% purity, a 72-kDa His-Tag-tagged fusion protein (Fig. 7A). We verified the identity of this protein by Western immunoblot analysis using rabbit anti-*M. soehngenii* Acs as the primary antibody (Fig. 7B). Using this purified 72-kDa Acs protein, we generated rabbit anti-*E. coli* Acs antibody, which we used for Western immunoblot analysis (Fig. 8) of lysates derived from cells which were wild type

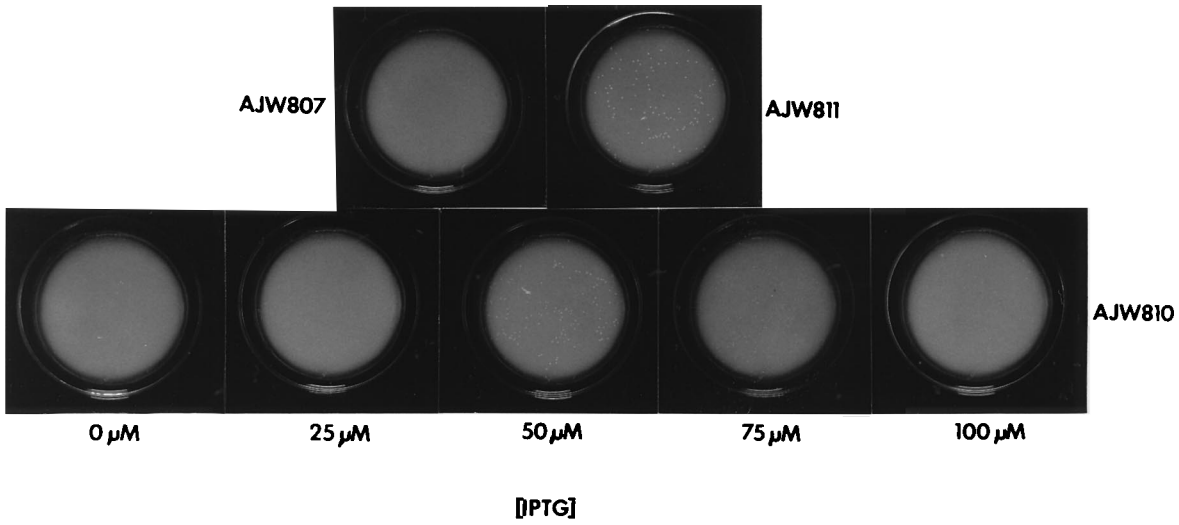

FIG. 6. Growth on acetate as the sole carbon source of cells deficient for Acs, Ack, and Pta (strain AJW807) transformed with either pSR10 (strain AJW811) or pSR31 (strain AJW810), plasmids that carry *acs* expressed by either its native promoter or the *lac* promoter, respectively. Cells were grown at 37°C in TB to an OD_{610} of ~0.3, washed and resuspended in PBS, spread onto M63 minimal plates supplemented with 10 mM acetate and the indicated concentration of IPTG, incubated at 37°C for 72 h, and photographed.

(strainCP875) or deficient for Acs (strain AJW803), Ack and Pta (strain CP911), or Acs, Ack, and Pta (strain AJW804) and grown at 37°C in M63 minimal medium supplemented with acetate (10 mM) and glycerol (2.5 mM) to an OD_{610} of ~0.2. Cells of strains that carried the wild-type *acs* allele, i.e., CP875 (Fig. 8, lane 1) and CP911 (lane 3), expressed a 72-kDa protein. In contrast, cells of strains that carried the deletion allele Δ*acs*::Km, i.e., AJW803 (Fig. 8, lane 2) and AJW804 (lane 4), did not.

Finally, we determined that the purified 72-kDa protein can activate acetate across a wide range of acetate concentrations (Fig. 9). This activity required the presence of CoA and was stable only if CoA (533 μM) was present during purification (data not shown).

DISCUSSION

We conclude that *E. coli* cells possess a single gene that encodes Acs and that it maps within the 92.3- to 92.4-min region of the chromosome, as proposed by Blattner et al. (5). The following observations support this hypothesis. (i) The open reading frame, as predicted by the reported sequence, encodes a 72-kDa protein which cross-reacts with polyclonal antibody specific for Acs isolated from an unrelated species, *M. soehngenii*. (ii) Cells that express Ack and Pta, but not the 72-kDa protein, grow poorly on low concentrations of acetate; those that express the 72-kDa protein, but not Ack and Pta, grow poorly on high concentrations of acetate; and those that do not express any of the three proteins do not grow on any amount of acetate. (iii) Relative to wild-type cells, those that express Ack and Pta, but not the 72-kDa protein, do not activate acetate as well; those which express the 72-kDa protein, but not Ack and Pta, display even less activity; and those that do not express any of the three proteins exhibit no observable activity at all. (iv) Plasmids that express the 72-kDa protein restore growth on acetate to cells deficient for all three proteins. (v) The 0.72-kb *Bcl*I-*Bcl*I probe derived from pSR9 hybridized to only those recombinant phages, λ638 and λ639, that carry the 92.3- to 92.4-min region of the chromosome.

The unrelated species *E. coli* and *Bacillus subtilis* each possess and utilize both the Acs and the Ack-Pta pathways (3, 6, 8, 15, 16, 41). In contrast, some prokaryotes, e.g., *Alcaligenes eutrophus* (49, 52) and the *Methanothrix* spp. (25, 37), and apparently all eukaryotes (29) utilize only the Acs pathway. Other prokaryotes, e.g., the *Methanosarcina* spp. (1, 12, 28, 31, 51), use exclusively the Ack-Pta pathway.

If other organisms survive perfectly well using only one acetate activation pathway, then why do cells of *E. coli* and *B. subtilis* possess both pathways? Cells of both organisms excrete acetate during exponential aerobic growth in the presence of

TABLE 2. Acetate-activating activity in tryptone-grown cells which were either wild type or impaired for acetate activation[a]

Strain	Sp act of cells at the indicated OD_{610} in acetate concn of:								
	2.5 mM			5.0 mM			10.0 mM		
	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6
CP875 (Ack^+ Pta^+ Acs^+)	1.1 ± 0.1	2.6 ± 0.1	3.1 ± 0.1	2.9 ± 0.2	4.6 ± 0.1	6.8 ± 0.1	5.8 ± 0.2	7.4 ± 0.1	7.6 ± 0.1
AJW803 (Ack^+ Pta^+ Acs^-)	1.2 ± 0.2	2.2 ± 0.0	2.2 ± 0.1	2.9 ± 0.0	3.8 ± 0.1	4.8 ± 0.1	5.3 ± 0.1	7.6 ± 0.3	6.9 ± 0.2
CP911 (Ack^- Pta^- Acs^+)	BLD[b]	0.1 ± 0.0	0.2 ± 0.0	BLD	<0.1	0.4 ± 0.1	BLD	1.0 ± 0.2	1.3 ± 0.1
AJW804 (Ack^- Pta^- Acs^-)	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD	BLD

[a] Cells were harvested at OD_{610}s of 0.2, 0.4, and 0.6 after growth at 37°C in TB. Crude lysates were prepared and Acs activities were measured as described in Materials and Methods. Mean specific activities and standard errors of the means of independent duplicate experiments are expressed as milliunits per milligram, where 1 mU of activity catalyzes the formation of 1 nmol of acetohydroxamate per min.

[b] BLD, below the level of detection, i.e., 0.01 mU mg^{-1}.

TABLE 3. Acetate-activating activity in acetate-grown cells which were wild type or impaired for acetate activation[a]

Strain	Sp act at acetate concn (mM) of:		
	2.5	5.0	10.0
CP875 (Ack^+ Pta^+ Acs^+)	2.3 ± 0.4	4.3 ± 0.4	7.1 ± 0.5
AJW803 (Ack^+ Pta^+ Acs^-)	1.8 ± 0.2	3.5 ± 0.4	6.2 ± 0.0
CP911 (Ack^- Pta^- Acs^+)	0.9 ± 0.0	1.1 ± 0.0	0.9 ± 0.1
AJW804 (Ack^- Pta^- Acs^-)	BLD[b]	BLD	BLD

[a] Cells were harvested at an OD_{610} of 0.2 after growth at 37°C in M63 salts medium supplemented with 50 mM acetate and 2.5 mM glycerol. Crude lysates were prepared and Acs activities were measured as described in Materials and Methods. Mean specific activities and standard errors of the means of four independent experiments are expressed as milliunits per milligram, where 1 mU of activity catalyzes the formation of 1 nmol of acetohydroxamate per min.

[b] BLD, below the level of detection, i.e., 0.01 mU mg^{-1}.

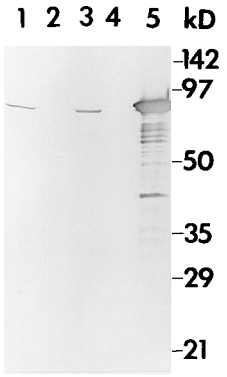

FIG. 8. Immunoblot analysis using rabbit anti-*E. coli* Acs polyclonal antibody after SDS-PAGE (10% polyacrylamide) of lysates from cells wild type for acetate activation (strain CP875 [lane 1]) or deficient for Acs (strain AJW803 [lane 2]), Ack and Pta (strain CP911 [lane 3]), or Acs, Ack, and Pta (strain AJW804 [lane 4]). Purified *E. coli* Acs (lane 5) was also used. Molecular mass standards are labeled in kilodaltons to the right of the gel.

excess carbon (11, 21, 23, 30, 38, 48). Acetate excretion (Fig. 5) and intracellular AcP accumulation by cells of *E. coli* throughout early-exponential-phase growth in tryptone broth depends on the presence of both Pta and Ack (39). Similarly, early-exponential-phase growth of *B. subtilis* cells in media containing high glucose concentrations results in both elevated *ackA* expression (15) and elevated Pta levels (40). Midway through exponential growth, cells of *E. coli* begin utilizing the acetate they had excreted previously, a process that requires Acs (6) (Fig. 5). Similarly, *B. subtilis* cells induce *acsA* expression as they enter stationary phase (14).

Whereas *B. subtilis* cells require Acs for growth or sporulation on acetate as the sole carbon source, *E. coli* cells can utilize either pathway to grow on acetate as the sole carbon source, albeit with significantly different affinities for acetate. Although *E. coli* cells which are wild type for both pathways grow about equally well across a 20-fold range of acetate concentrations, i.e., from 2.5 to 50 mM, those deficient for either pathway grow poorly at one end or the other of that range. Ack binds acetate poorly (K_m = 7 mM) (13), while Acs binds with much higher affinity (K_m = 0.2 mM) (6). Thus, one might predict that, on low acetate concentrations, cells which express only Ack and Pta would grow poorly, while those that express only Acs would grow well. We have shown this prediction to be correct. One also might expect that cells which express only Acs would grow about as well on higher concentrations of acetate as they do on lower ones. However, high concentrations seemingly inhibit growth of these cells. Such acetate concentrations are much larger than that required to achieve V_{max}. Perhaps Acs cannot process acetate sufficiently fast to reduce the toxicity associated with high acetate concentrations. Perhaps possession of both acetate activation pathways permits *E.*

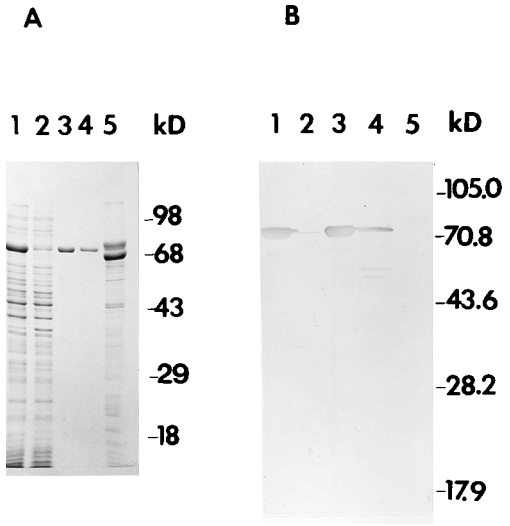

FIG. 7. Purified *E. coli* Acs isolated from strain BL21(DE3) transformed with a plasmid (pSR30) which expresses Acs from the T7 promoter. Lane 1, whole-cell extract prior to loading onto the nickel column; lane 2, column flowthrough; lane 3, column eluent; lane 4, column eluent with His-Tag cleaved with thrombin; lane 5, purified Acs from *Saccharomyces cerevisiae* mitochondria (Sigma). Molecular mass standards are labeled in kilodaltons to the right of the gels. (A) SDS–10% polyacrylamide gels stained with Coomassie blue. (B) Immunoblot analysis using rabbit anti-*M. soehngenii* Acs following SDS-PAGE (10% polyacrylamide). Note (lane 4) that cleavage by thrombin of the His-Tag–Acs fusion protein results in the spurious production of two additional, smaller products (molecular masses between 50 and 60 kDa).

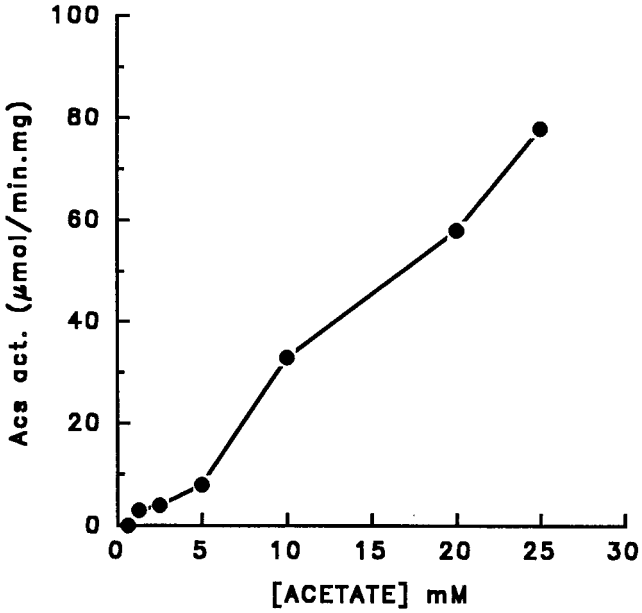

FIG. 9. Activity of purified *E. coli* Acs plotted as a function of acetate concentration. The purification and assay conditions are described in Materials and Methods. In the absence of CoA, no activity was detected at all concentrations of acetate tested. Acs act., Acs activity.

coli cells to survive in environments that contain widely varying concentrations of acetate.

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